Supporting Information

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SI Materials and Methods

Growth Conditions and Generation of Yeast Variants. Yeast were grown in full media YPD/YPS/YPG/YPGal [1% yeast extract, 2% (wt/vol) peptone, and 2% (wt/vol) glucose/2% (wt/vol) sucrose/3% (wt/vol) glycerol/2% (wt/vol) galactose, respectively] or in minimal media that contained 0.5% ammonium sulfate and 0.17% yeast nitrogen base, supplemented with suitable nutrients and a carbon source. For proteasome inhibition with MG132 or PS341 (75 μM), the modified minimal medium without ammonium sulfate but with 0.1% proline and 0.003% SDS was used as described previously (1).

The $Tom40_{CFREE}$ and $Tom40_{C130/C138}$ variants were prepared on the template of the wild-type Tom40-containing plasmid (pFL39) by removal of native cysteine codons (C165W, C326A, C341S, and C355F) followed by introduction of cysteine codons at new positions (N130C and S138C) (2). The strains expressing Tom40 variants were generated by plasmid shuffling similar to the corresponding wild-type strain (2). For production of b_2-$Mia40_{core}$ in wild-type cells that also express native Mia40, yeast were transformed with centromeric plasmids pGB9310 (pFL39-b_2-$Mia40_{core}$; 47) or pPB13.1 (pRS416-b_2-$Mia40_{core}$; 166) for tryptophane or uracil selection, respectively, with the fusion protein placed under the control of native Mia40 promoter and terminator sequences (3). The plasmid with b_2-$Mia40_{core}$ was also used as a template for generation of cysteine to serine variants: pGB9477 (pFL39-b_2-$Mia40_{core}$-C1S; 48), pPB11.1 (pFL39-b_2-$Mia40_{core}$-C2S; 49), pPB12.4 (pFL39-b_2-$Mia40_{core}$-C3S; 50), pPB16.2 (pRS416-b_2-$Mia40_{core}$-C3S; 168), pGB9479 (pFL39-b_2-$Mia40_{core}$-C4S; 51), and pPB17.1 (pRS416-b_2-$Mia40_{core}$-C4S; 169). The cysteine residues are numbered as in Böttinger et al. (4). For production of FLAG-tagged proteins, yeast were transformed with the pESC-URA plasmid (Ambion) encoding $Cox12_{FLAG}$ (pAG3; 55), $Pet191_{FLAG}$ (pAG1; 53), and $Mix17_{FLAG}$ (pAG2; 54), with the fusion protein under the control of inducible *GAL10* promoter. Additionally, based on pAG1, the construct encoding head-to-tail dimer of Pet191 (Pet191_$Pet191_{FLAG}$) was created (pPB22.1; 170). To induce the *GAL10* promoter, addition of 0.5% galactose to the glycerol-containing medium was used. The b_2-$Cox12_{FLAG}$–encoding plasmid (pPB23.1; 212) was prepared based on pPB13.1 with $Cox12_{FLAG}$ recloned from pAG3.

Cell Fractionation and Mitochondrial Procedures. For fractionation, the equivalent of 40 OD_{600} units of yeast cells grown at respiratory conditions at 37 °C was used. The fractionation procedure was described previously (1). For each fraction, an equivalent of 0.8 OD_{600} units of cell extract was tested. Yeast mitochondria were isolated using the standard method with differential centrifugation (5) and were stored at –80 °C in the sucrose/Mops (SM) buffer (250 mM sucrose and 10 mM Mops/KOH, pH 7.2). Human mitochondria were isolated from HEK293 cells cultured at 37 °C under 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) FBS and 2 mM L-glutamine. For mitochondria isolation, cells were collected by scrapping and homogenized in a Dounce homogenizer in the isolation buffer [300 mM trehalose, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.1% BSA (wt/vol), 2 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM Hepes/KOH, pH 7.7] (6). Cell debris was removed by centrifugation at 1,000 × *g* for 10 min. Mitochondria were isolated by centrifugation at 10,000 × *g* for 15 min, and the mitochondrial pellet was resuspended in the isolation buffer without BSA and directly used without storage. Protein content was quantified using the Bradford assay. To generate mitoplasts, mitochondria were incubated for 30 min on ice in the hypoosmotic buffer indicated in the figure legends (Figs. S2 *A* and *B* and S4 *A* and *B*). If indicated, samples were split into halves and further incubated with or without 10 μg/mL of proteinase K for 15 min. Proteinase K activity was inhibited by addition of phenylmethanesulfonyl fluoride (PMSF, 2 mM). Mitochondria or mitoplasts were then reisolated by centrifugation. Supernatant fractions were precipitated with 10% (wt/vol) TCA.

Import of Radiolabeled Precursor Proteins. The [^{35}S]methionine-labeled precursor proteins were synthesized using the rabbit reticulocyte TNT Quick Coupled Transcription/Translation kit (Promega). The import of radiolabeled precursors into isolated yeast mitochondria was performed at 25 °C in the import buffer [250 mM sucrose, 5 mM $MgCl_2$, 80 mM KCl, 10 mM Mops/KOH, 5 mM methionine, 3% (wt/vol) BSA, 10 mM KH_2PO_4/K_2HPO_4, pH 7.2] supplemented with 2 mM ATP and 2 mM NADH. Reactions were stopped by cooling on ice and dissipation of the IM potential by addition of 1 μM valinomycin, 20 μM oligomycin, and 8 μM antimycin. To remove nonimported proteins, samples were treated with 25 μg/mL proteinase K for 15 min on ice. Reactions were stopped by addition of 2 mM PMSF. After centrifugation and washing with SM buffer, the samples were denatured in reducing (50 mM DTT) Laemmli buffer and analyzed by SDS/PAGE. Radioactive signal was detected by digital autoradiography.

Miscellaneous. Total cellular protein extracts were prepared by alkaline lysis (7). For immunodetection, enhanced chemiluminescent signals were detected by ImageQuant LAS4010 (GE Healthcare) or X-ray films. For densitometry, ImageQuant TL (GE Healthcare) software was used. In some figures, nonrelevant gel parts were excised digitally. The images were processed using Photoshop CS4 (Adobe).

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3. Chacinska A, et al. (2008) Mitochondrial biogenesis, switching the sorting pathway of the intermembrane space receptor Mia40. *J Biol Chem* 283(44):29723–29729.
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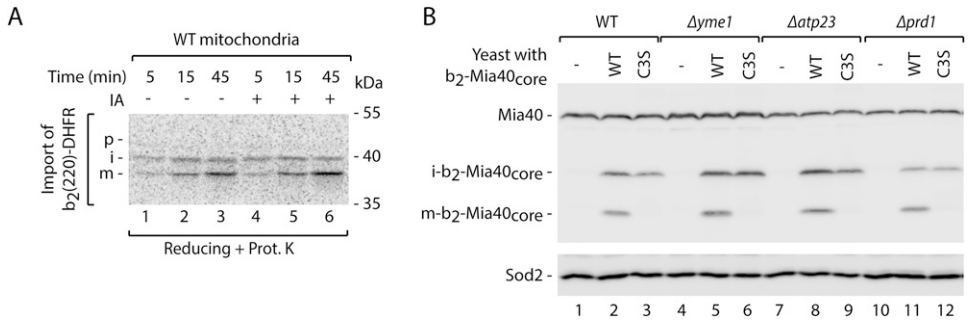

Fig. S1. (*A*) Import of [^{35}S]b_2(220)-DHFR into WT mitochondria with or without IA (50 mM). Blocking of thiol groups does not affect the presequence-dependent import into mitochondria. (*B*) Cellular levels of b_2-Mia40$_{core}$ variants in WT, *Δyme1*, *Δatp23*, and *Δprd1* strains were not changed. Proteins were analyzed by autoradiography (*A*) or SDS/PAGE and immunodetection (*B*). i, intermediate; m, mature; p, precursor; WT, wild type.

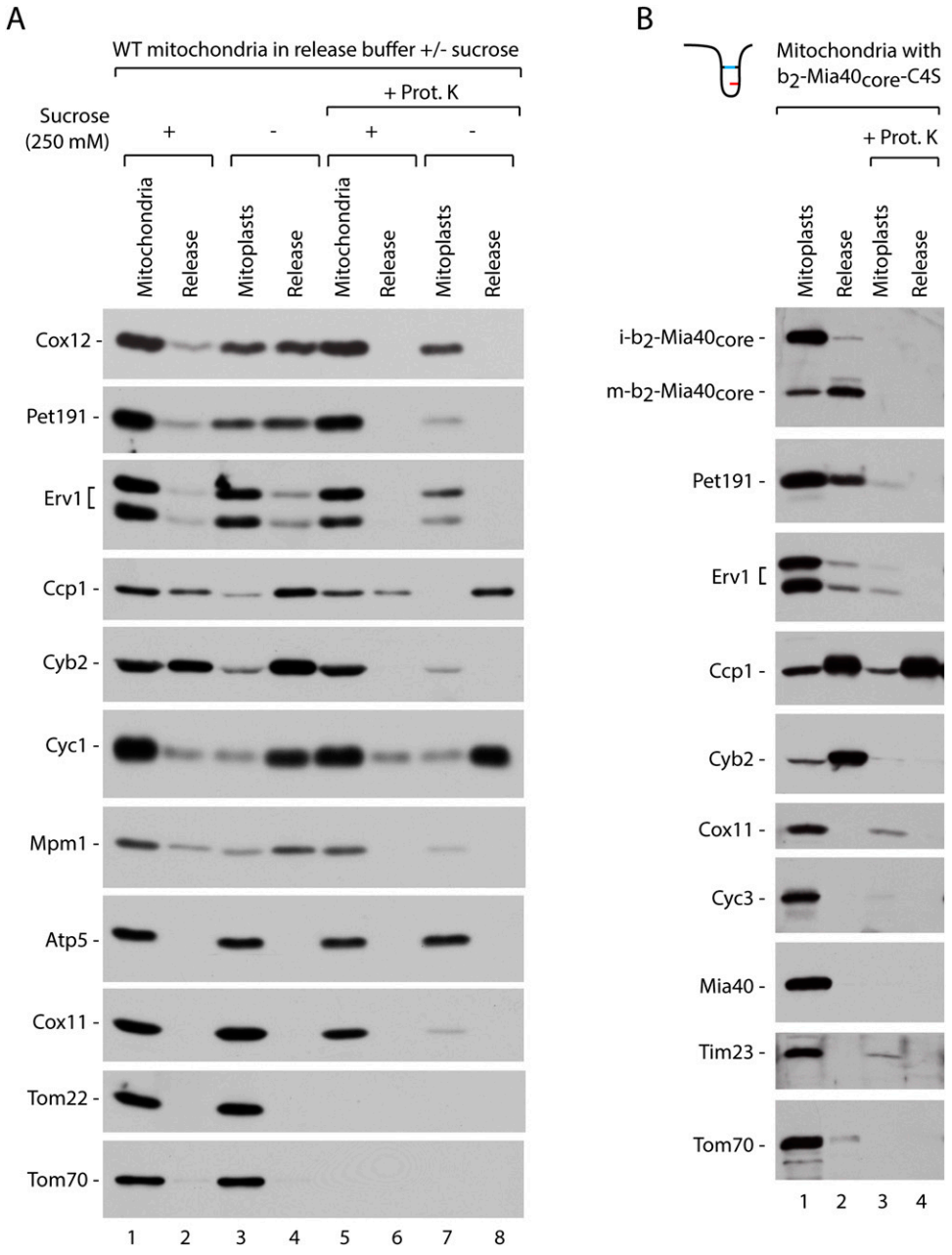

Fig. S2. *In organello* validation of protein release susceptibility and protease sensitivity. (*A*) Protein levels in isolated WT yeast mitochondria or mitoplasts and the corresponding supernatant fractions (release) upon incubation in the release buffer. Mitoplasts were generated by hypoosmotic swelling in the release buffer without sucrose. Samples were treated with or without proteinase K as indicated. Ccp1, Cyb2, Cyc1, and Mpm1 proteins were efficiently released from mitoplasts. Cox12, Pet191, and Erv1 proteins were partially released from mitoplasts. Cox12, Pet191, Erv1, Cyb2, Mpm1, and Cox11 proteins were sensitive to protease treatment only in mitoplasts, and Tom22 and Tom70 were sensitive to protease treatment in intact mitochondria and mitoplasts. (*B*) Protein levels in mitoplasts generated from mitochondria of WT yeast expressing b_2-Mia40$_{core}$-C4S and supernatant fractions (release). Mitoplasts were generated by the hypoosmotic swelling in the SM buffer containing 25 mM sucrose. Samples were treated with or without proteinase K, as indicated. The m-form, but not i-form, of b_2-Mia40$_{core}$-C4S was efficiently released from mitoplasts similar to Pet191, Ccp1, and Cyb2 proteins. Both forms of b_2-Mia40$_{core}$-C4S were sensitive to proteinase K treatment, similar to Pet191, Erv1, Cyb2, Cox11, Cyc3, Mia40, Tim23, and Tom70 proteins. In *A* and *B*, protein extracts were analyzed by SDS/PAGE and immunodetection. WT, wild type.

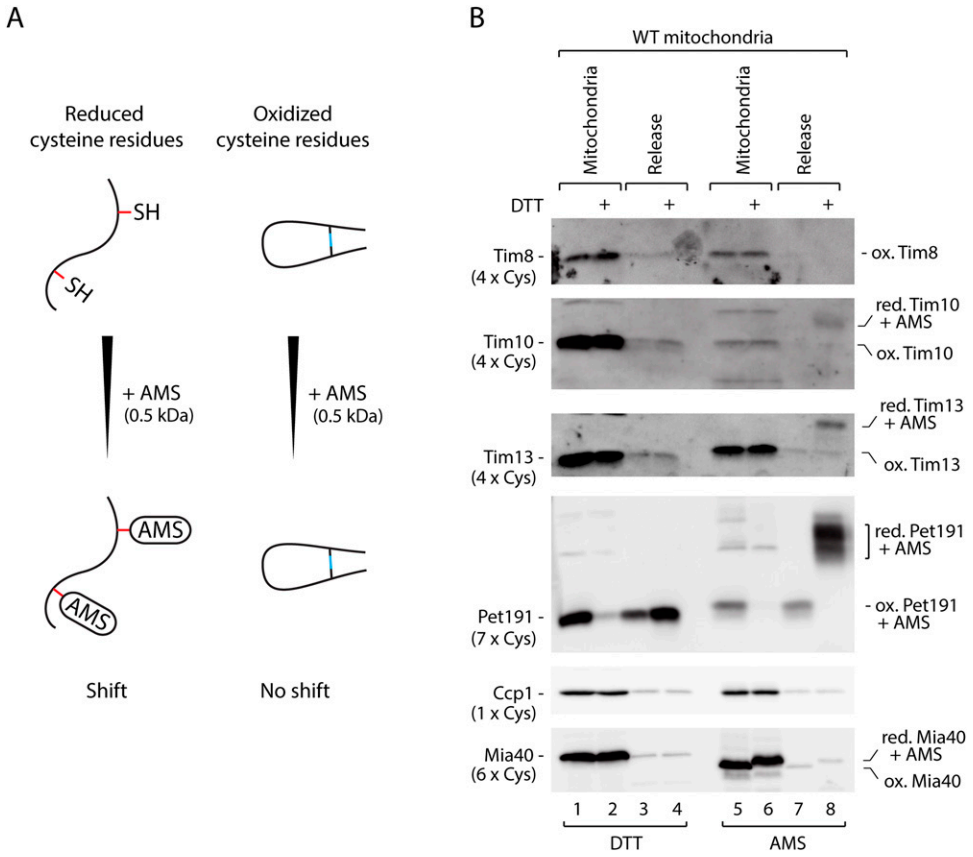

Fig. S3. Redox state of proteins released from mitochondria upon DTT treatment. (*A*) Schematic representation of thiol modification by AMS used to determine protein redox state. Only the reduced thiol groups react with AMS, resulting in the increased molecular weight and shift in protein migration on the SDS/PAGE. The modification was applied in the 2× Laemmli buffer. (*B*) Protein levels and redox states in isolated WT mitochondria and the corresponding supernatants (release) upon treatment with DTT. Pet191 was efficiently released from mitochondria upon DTT treatment, and the released protein was modified with AMS, indicating its reduced state. In contrast, Tim8, Tim10, and Tim13 were largely resistant to DTT treatment, with only a minor fraction found to be reduced and released. Protein extracts were analyzed by SDS/PAGE and immunodetection. Number of cysteine residues present in the mature protein is given in parentheses. Ox, oxidized; red, reduced; WT, wild type.

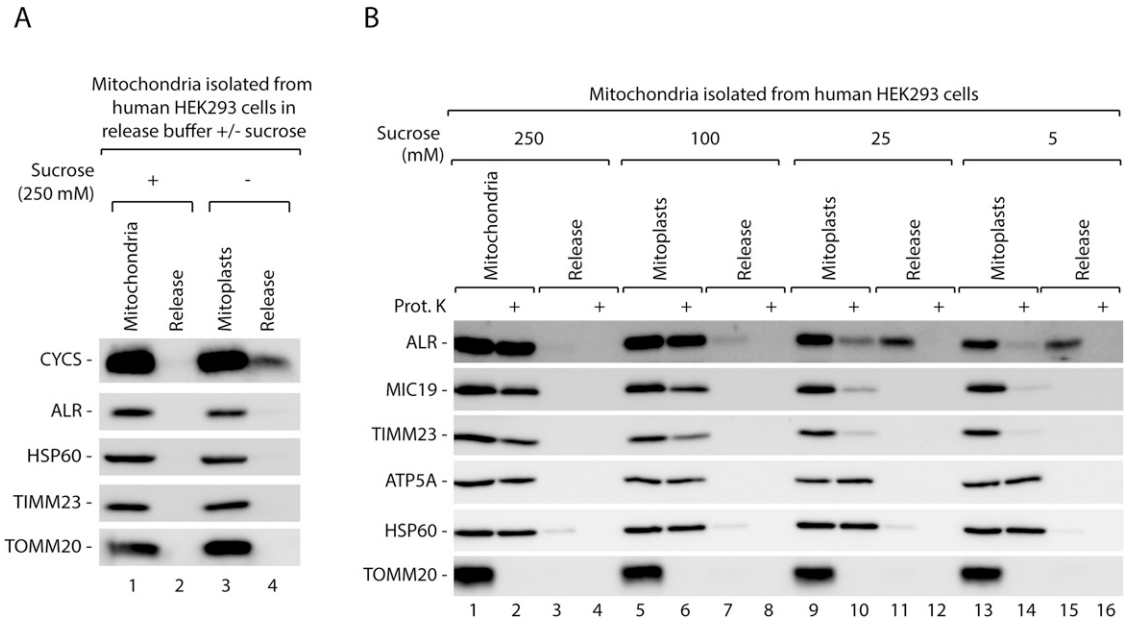

Fig. S4. *In organello* validation of human mitochondrial protein release susceptibility and protease sensitivity. (*A*) Protein levels in isolated human HEK293 mitochondria, mitoplasts, and the corresponding supernatant fractions (release) upon incubation in the release buffer. Mitoplasts were generated by hypoosmotic swelling in the release buffer without sucrose. The CYCS protein was released from mitoplasts but not mitochondria. (*B*) Protein levels in human HEK293 mitochondria, mitoplasts, and supernatants (release). Mitochondria were subjected to the hypoosmotic swelling in 20 mM Hepes buffer, pH 7.4 with 100, 25, or 5 mM sucrose. Samples were treated with or without proteinase K as indicated. In the case of 25 mM and 5 mM sucrose-treated samples (mitoplasts), ALR was found in the release fraction. In the mitoplast samples, ALR, MIC19, and TIMM23 were sensitive to proteinase treatment, whereas TOMM20 was sensitive to proteinase treatment in mitochondria and mitoplasts. In *A* and *B*, protein extracts were analyzed by SDS/PAGE and immunodetection.

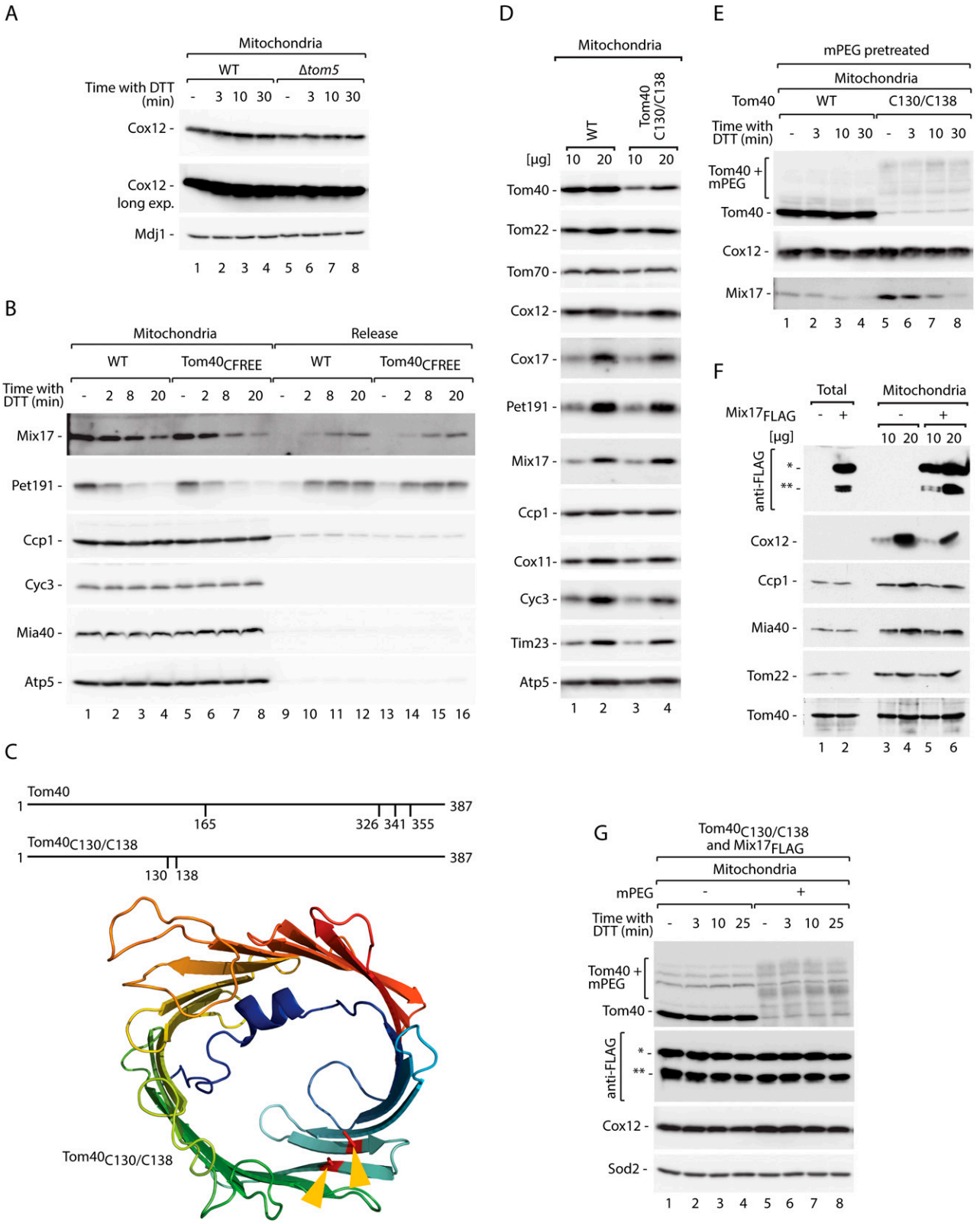

Fig. S5. Characterization of mitochondria with Tom40 variants. (*A*) Protein levels in the WT and *Δtom5* mitochondrial fractions upon treatment with DTT correspond to the release fractions shown in Fig. 4*C*. (*B*) Comparison of protein levels in mitochondria with wild-type Tom40 or $Tom40_{CRFEE}$ and the corresponding supernatants (release) upon treatment with DTT for the indicated time. (*C*) A schematic representation of *S. cerevisiae* Tom40 and $Tom40_{C130/C138}$, with marked positions of cysteine residues at the top. The $Tom40_{C130/C138}$ model with cysteine residue sulfhydryl groups (red) marked with yellow arrowheads in the lumen of the channel at the bottom. The Tom40 homology model generated by Qiu et al. (1) was generated using PyMOL after the side chains of residues 130 and 138 were replaced by cysteines using Coot without changing the main chain (2). (*D*) Protein levels in mitochondria isolated from strains

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expressing wild-type Tom40 or $Tom40_{C130/C138}$. (*E*) Protein levels in the WT and $Tom40_{C130/C138}$ mitochondrial fractions, pretreated with mPEG followed by the treatment with DTT for the indicated time, correspond to the release fractions shown in Fig. 4*E*. (*F*) Analysis of $Mix17_{FLAG}$ in cellular protein extracts and isolated mitochondria. (*G*) Protein levels in the mitochondria with $Tom40_{C130/C138}$ and $Mix17_{FLAG}$, with or without pretreatment with mPEG, upon treatment with DTT for the indicated time, correspond to release fractions shown in Fig. 4*F*. In *A*, *B*, and *D*–*G*, protein extracts were analyzed by SDS/PAGE and immunodetection. WT, wild type; *, full-size $Mix17_{FLAG}$; **, $Mix17_{FLAG}$ degradation product.

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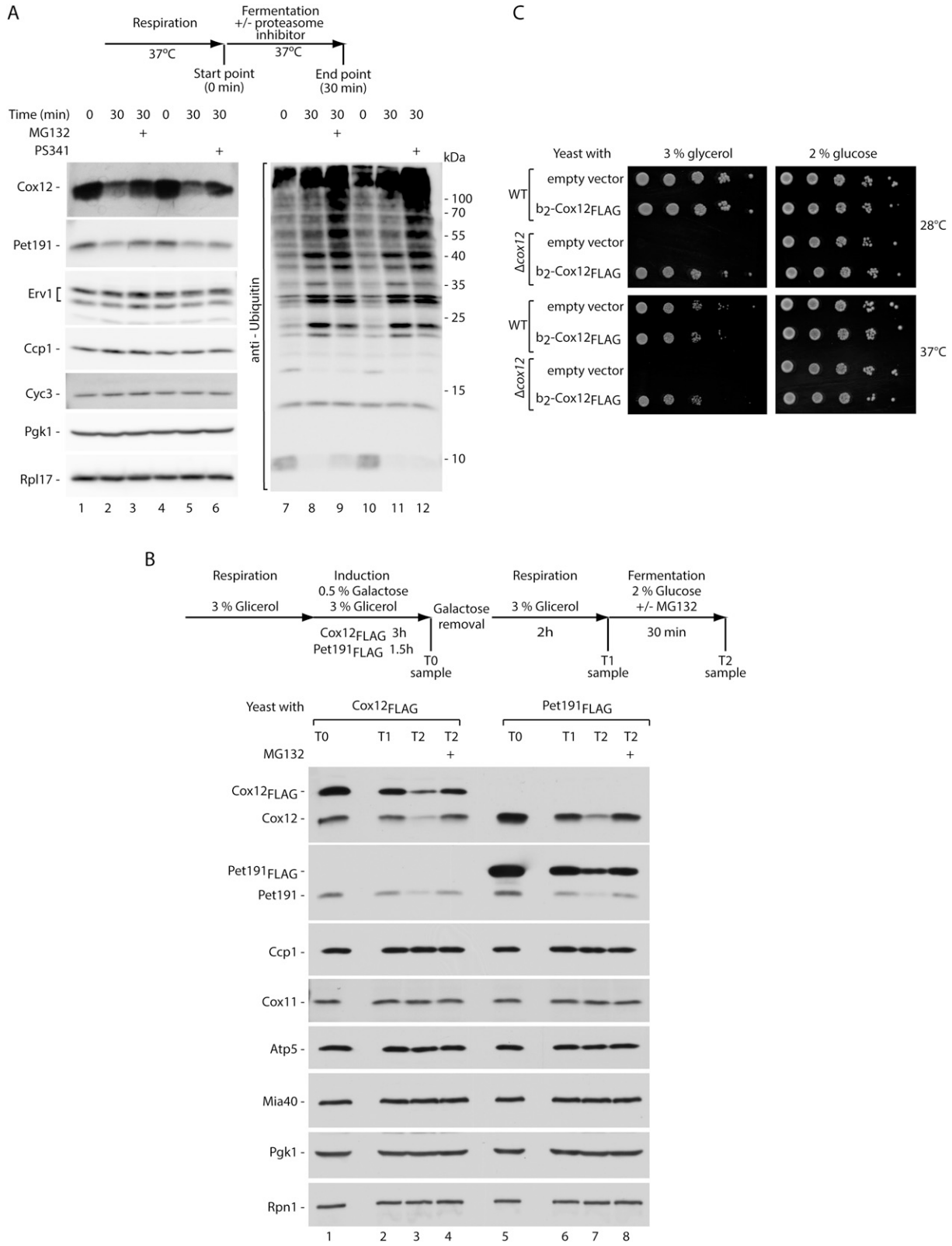

Fig. S6. The respiration-to-fermentation shift induces IMS protein susceptibility to proteasomal clearance. (*A*) Cellular protein levels upon shift from respiration to fermentation with or without proteasome inhibitor. Final concentration of the proteasome inhibitors MG132 and PS341 was 75 μM. Experimental scheme is provided at the top. (*B*) Cellular levels of $Cox12_{FLAG}$ and $Pet191_{FLAG}$ upon expression induction pulse (T0), after 2 h chase without induction (T1) and upon shift from respiration to fermentation with or without MG132 (T2). Experimental scheme is provided at the top. (*C*) Growth test of WT and Δ*cox12* cells transformed with the b_2-$Cox12_{FLAG}$–encoding plasmid or empty vector. Serial 10-fold dilutions of yeast cells were analyzed on minimal medium with carbon source as indicated. In *A* and *B*, protein extracts were analyzed by SDS/PAGE and immunodetection. WT, wild type.

Table S1. *S. cerevisiae* strains used in this study

Strain (laboratory ID no.)	Genotype	Reference
YPH499 (524)	*MAT*a, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801*	(1)
Erv1-2 (235)	*MAT*a, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, erv1::ADE2* [pFL39-erv1-2]	(2)
Erv1-2int (359)	*MAT*a, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, erv1::erv1-2*	(3)
Tom40 (772) WT for $tom40_{C130/C138}$ and $tom40_{CFREE}$	*MAT*a, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63 lys2-801, tom40::ADE2* [pFL39-TOM40]	(4)
$tom40_{CFREE}$ (771)	*MAT*a, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63 lys2-801, tom40::ADE2* [pFL39-TOM40-C165W/C326A/C341S/C355F]	(4)
$tom40_{C130/C138}$ (769)	*MAT*a, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63 lys2-801, tom40::ADE2* [pFL39-TOM40-N130C/S138C/C165W/C326A/C341S/C355F]	(5)
BY4741 (755)	*MAT*a, *his3Δ 1, leu2Δ 0, met15Δ 0, ura3Δ 0*	Euroscarf
Δ*cox12* (722)	*MAT*a, *his3Δ 1, leu2Δ 0, met15Δ 0, ura3Δ 0, YLR038c::kanMX4*	Euroscarf
Δ*tom5* (569)	*MAT*a, *his3Δ 1, leu2Δ 0, met15Δ 0, ura3Δ 0, YPR133w-a::kanMX4*	Euroscarf
Δ*rad6* (861)	*MAT*a, *his3Δ 1, leu2Δ 0, met15Δ 0, ura3Δ 0, YGL058w::kanMX4*	Open Biosystems
Δ*ate1* (626)	*MAT*a, *his3Δ 1, leu2Δ 0, met15Δ 0, ura3Δ 0, YGL017w::kanMX4*	Euroscarf
Δ*yme1* (671)	*MAT*a, *his3Δ 1, leu2Δ 0, met15Δ 0, ura3Δ 0, YPR024w::kanMX4*	Euroscarf
Δ*atp23* (700)	*MAT*a, *his3Δ 1, leu2Δ 0, met15Δ 0, ura3Δ 0, YNR020c::kanMX4*	Euroscarf
Δ*prd1* (741)	*MAT*a, *his3Δ 1, leu2Δ 0, met15Δ 0, ura3Δ 0, YCL057w::kanMX4*	Euroscarf

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4. Qiu J, et al. (2013) Coupling of mitochondrial import and export translocases by receptor-mediated supercomplex formation. *Cell* 154(3):596–608.
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